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Research Article

Combined 624-nm and 850-nm illumination at low rates leads to enhanced inhibition of *Candida albicans*

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ABSTRACT

Background: To determine whether combinations of red and infrared light could improve inhibition of *Candida albicans* and whether combining wavelengths and altering rate of energy delivery could prevent the formation of resistance to light energy.
Methods: *C. albicans* was tested because of the common appearance in human skin and mucous membrane infections. The organism was treated *in vitro* with a combination of 624-nm (red) and 850-nm (infrared) light emitted from a supraluminous diode (SLD) array. Doses of 9, and 30 J/cm^2 were used. Rate of energy delivery was also manipulated. Colony counts were performed and compared to untreated controls using Student *t* tests and one-way ANOVA with Tukey *post hoc* analysis.
Results: The combination of 624 and 850-nm light energy at 30 J/cm^2 was an effective ($p \leq 0.05$) inhibitor of *C. albicans* across all seven stages of the experiment. The combination of 624 and 850-nm wavelengths produced a maximum kill rate [{control – treated / control} X 100] of 76.24% and an average kill rate of 54% across the seven stages of the experiment.
Conclusions: A Combination of 624-nm and 850-nm light from an SLD array can inhibit the growth of *C. albicans in vitro*. Altering delivery rate of the energy can delay resistance formation in this organism.

Keywords: Red and infrared light, *C. albicans*, Fungicidal effect

INTRODUCTION

Emerging antimicrobial resistance trends represent a significant challenge for all clinicians treating injuries to the skin.[1,2] Pharmaceutical agents have historically been the treatment of choice for infectious diseases; however, antimicrobial resistance poses a threat to the long term effectiveness of pharmaceuticals.[3] The looming threat of antibiotic resistance has sparked a growing interest in validating alternative antimicrobial techniques, particularly those that are less likely to facilitate the development of microbial resistance. Bush et al. have encouraged the search for novel non-antibiotic techniques that provide prevention and protection against infectious disease.[4] We hypothesize that visible and infrared light may offer effective, alternative treatment options for addressing infectious organisms and their inherent ability to develop resistance to various therapies.

The use of electromagnetic waves to produce an antimicrobial outcome is not novel. Ultraviolet C light, which ranges from 180-290 nm, has the ability to destroy microorganisms.[5] A 100% kill rate of *E. coli* has been reported in association with treatment using 265 nm (UVC) light.[6] Unfortunately, UVC light can be harmful to mammalian cells.[7] DNA lesions have been observed directly after a 6.48 J/cm^2 UVC exposure.[5]

Photodynamic therapy (PDT) is another technique that employs electromagnetic waves in the treatment of microorganisms. PDT includes the introduction of a photosensitizing dye. This dye can lead to the production of cytotoxic species.[8] Methylene blue and red light (660 nm) has been shown to effectively inhibit *Enterococcus faecalis*.[9] However, it has been pointed out that it can be difficult to introduce photosensitizers to some bacteria.[1] Additionally, some photosensitizers are not particularly selectively sensitive for bacteria versus host tissue.[1]

Blue light (405nm, 415nm and 470nm) is an effective inhibitor of *Staphylococcus aureus*,[10,11,12] *Staphylococcus epidermis*,[12] *Mycobacterium smegmatis*[13,14] and *Pseudomonas aeruginosa*.[1,10,11] Compared to the risks of cell damage associated with UVC and to the photosensitizer specificity issues sometimes seen with PDT, blue light is a safe, effective and less potentially problematic alternative treatment option. No significant damage to the skin of mice occurred when treated with blue light[1] at doses as high as 55.8 J/cm^2 and no significant change in osteoblast morphology, function or viability occurred when exposed to 405 nm light at 18 J/cm^2.[12]

Recently, *S. aureus* was shown to have the ability to develop resistance to blue light when treatment was extended past five generations. Alterations in wavelength combinations and rate of energy delivery seem to significantly impede this resistance formation (J.S. Guffey, W. Payne, K. Martin and C. Dodson, submitted for publication).

PDT and UVC produce fungicidal effects when used to treat *Candida albicans*.[5,16,17] Various combinations of photosensitizers and laser treatments have produced a significant kill rate for *C. albicans*, with the combination of methylene blue and red light demonstrating a significant kill rate across multiple studies.[18,19] Barbario et al. recently demonstrated a significant kill rate of *C. albicans* when exposed to a combination of 0.05 mg/mL toluidine blue O and 18 J/cm^2 of red light.[20] In an *in vivo* study, Dai et al. found UVC light (254 nm) to be superior to a topical antifungal drug in treating *C. albicans*.[5] Based on these results, it can be said that *C. albicans* can be treated with various wavelengths when appropriate photosensitizers are included.

Red light (624nm) has been demonstrated to inhibit colony formation in yeasts and fungi (J.S. Guffey, W. Payne, L. James, Z. Qian, and C. Dodson, submitted for publication). In this study, blue light wavelengths had little effect on *C. albicans*, but red light did produce a moderate, but statistically significant kill rate. The response of *C. albicans* to various wavelengths of light suggests potential for the use of light therapy to treat fungal infections such as candidiasis.

The purpose of this investigation was to determine whether combinations of red and infrared light could improve inhibition of *C. albicans* and whether combining wavelengths and altering rate of energy delivery could prevent the formation of resistance to light energy. We come to this question accepting the premise that reactive oxygen species (ROS) are source of the inhibition of the organism.[15] Whether this production of ROS is possible without the administration of a photosensitizing dye was not measured in our study, but we assumed a reduction in post-irradiation colony forming units (CFU) would be evidence of its production. Our decision to combine red and infrared (IR) wavelengths was fostered by our experience[10] of enhanced inhibition in bacteria when blue and IR wavelengths were combined.

METHODS

The organism used for this study was *C. albicans* (ATCC 14053), an organism that grows well in ambient air on nutritive media. Sabouraud dextrose agar (Difco, Detroit, USA), a moderately selective medium formulated to grow fungi, was chosen as a growth medium. The acidic pH (5.6) of the medium and the high concentration of dextrose are inhibitory for many species of bacteria. The organism was incubated at 37° C for a period of 20 hours. Use of a 20-hour-old culture is standard microbiological practice and serves to minimize the lag time for new growth.[21]

Using a sterile cotton-tipped swab, material was removed from the Sabouraud dextrose agar (SDA) and added to sterile deionized water to form a suspension equivalent to a 0.5 McFarland Standard (1.5 X 10^8 CFU/ml). The suspension was then diluted 1/1000 using 100 microliter automatic pipettes for purposes of accuracy and reproducibility. All dilutions were made immediately before the treatment with light. The dilution was then placed in a 60 X 15 mm sterile, polystyrene petri dish and light was delivered to the dilution in this container. The light source was brought into close proximity (as near as possible without contacting the suspension) to the dilution and irradiation was accomplished. After light exposure, using a 10 microliter automatic pipette, an aliquot of the 1/1000 dilution of *C. albicans* was inoculated onto SDA in 60 X 15 mm sterile, polystyrene petri dishes. The diluted yeast suspension was applied to the surface of the SDA plates in a star-streak pattern to enable colony counts to be performed after approximately 24 hours of incubation.

For this experiment, we chose to illuminate the cultures using a pair of SLD light pads that emitted a band of light focused around the primary wavelengths of 624-nm and 850-nm (Dynatron® 705^{Plus} Solaris™). See Table 1 for spectrum details of the SLD light source used in this study. The pads consisted of a 353 cm^2 illuminating surface area comprised of 176 SLDs with a maximum power output of 5160 mW. Dose was calculated in J/cm^2. Since output for the pad was held constant, adjustment in time of irradiation provided the dose (9 J/cm^2 or 30 J/cm^2).

Table 1: Spectral details of SLD light source.

Wavelength (nm)	Peak Wavelength (nm)	Dominant Wavelength (nm)	50% Output Low Side (nm)	50% Output High Side (nm)	Total Power Output (mW) @ 10 J/cm^2
624	640	624	620	650	
850	860	850	830	870	
624 & 850					4128

nm – nanometers
mW – milliwatts

The experiment was comprised of seven stages. Stage one was achieved by exposing the above described suspension of *C. albicans* to a combination of 624-nm and 850-nm light at 9 and 30 J/cm^2. The rate of the delivery of light energy was 17 mW/cm^2 for the first 5 stages. After 20 – 24 hours of incubation, colony forming units (CFUs) were counted. A subculture was taken from the plates of the most effective stage-one dose. A new suspension was made from that subcultured sample and used for stage two. This process was repeated through seven stages. Both 9 and 30 J/cm^{2}were used at each stage, but in the later stages of the experiment (stages 6 and 7) the rate of delivery was reduced by 50% to determine whether a declining effectiveness could be reversed.

Statistical Analysis

Data were analyzed using paired Student t-tests and a One-way ANOVA. Post hoc for the ANOVA was performed using Tukey's Honest Significant Difference. SPSS 20 was the software package employed for the data analysis.

RESULTS

The combination of 624 and 850-nm light energy at 30 J/cm^2 was an effective (student t-test) ($p \leq 0.05$ and a positive kill rate) inhibitor of *C. albicans* across all seven stages of the experiment. With regard to 9 J/cm^2, stages 5, 6 and 7 failed to produce a statistically significant kill rate. Table 2 displays this data in detail.

One-way ANOVA was performed and demonstrated that there was a significant difference across the stages (F = 19.422, df = 13, 56, p = 0.000). The post hoc analysis revealed that the differences across the stages lay primarily with the loss of effectiveness of 9 J/cm^2 in stages 5, 6 and 7. Figure 1 graphically demonstrates this point.

Table 2: Kill rate effect of combined 624 and 850-nm light on C. albicans by stage.

Stage	N	Dose (J/cm^2)	Rate (mW/cm^2)	$\bar{x}$ CFU (Control)	$\bar{x}$ CFU (Treated)	Kill Rate %	p value
1	5	9	17	17.6	5.8	67.04	0.003
	5	30	17	17.6	5.4	69.32	0.002
2	5	9	17	20.2	14	30.69	0.027
	5	30	17	20.2	4.8	76.23	0.000
3	5	9	17	20.0	9.6	52.00	0.000
	5	30	17	20.0	9.8	51.00	0.002
4	5	9	17	20.6	14.2	31.07	0.005
	5	30	17	20.6	12.4	39.80	0.011
5	***5***	***9***	***17***	***22.4***	***28.2***	***-25.89***	***0.079***
	5	30	17	22.4	14.8	33.92	0.000
6	***5***	***9***	***8.5***	***19***	***16.8***	***16.84***	***0.067***
	5	30	8.5	19	7.0	63.15	0.000
7	***5***	***9***	***8.5***	***20.6***	***25.6***	***-24.27***	***0.019***
	5	30	8.5	20.6	11.6	43.69	0.002

Bolded and Italicized Rows Were Ineffective Doses / Conditions
CFU – Mean Colony Forming Units

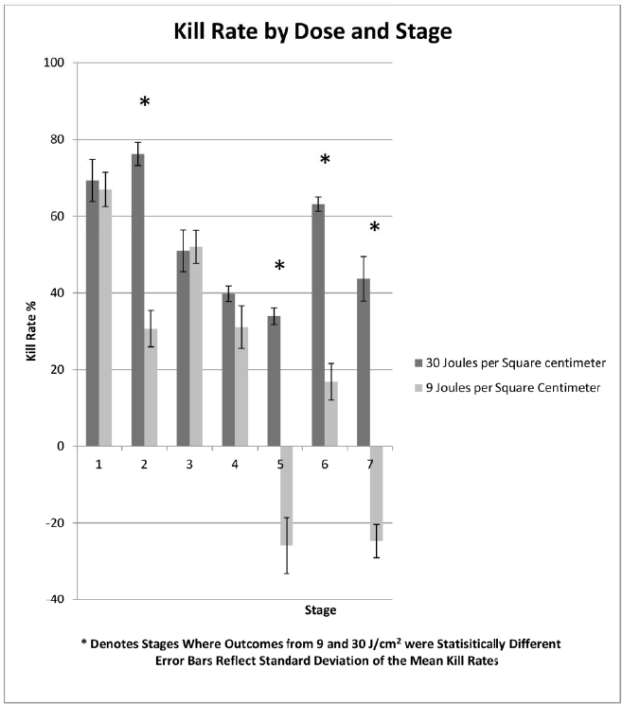

Figure 1: Kill rate by dose and stage: *C. albicans.*

Also important to note is that, with regard to the 30 J/cm^2 dose, stages 1, 2, 3 and 4 were not statistically different from stage 6 and 7.

DISCUSSION

The combination of red (624-nm) and infrared (850-nm) light energy is an effective inhibitor of *C. albicans*. We have already found that red light (624-nm) alone can inhibit *C. albicans*, but the rate of inhibition was not as strong (approximately 30% kill rate) as we saw in this experiment (J.S. Guffey, W. Payne, L. James, Z. Qian, and C. Dodson, submitted for publication). de Sousa et al reported that *C. tropicalis* had a native susceptibility to 685-nm light.[17] *C. tropicalis* is closely related to *C. albicans* and this may be why we saw some degree of inhibition using only 624-nm light in the unpublished work mentioned above. The combination of 624 and 850-nm wavelengths produced a maximum of 76.24% kill rate and an average kill rate of 54% across the seven stages of the experiment. While these rates if inhibition are statistically significant (Stages 1 – 4), the trend toward loss of effectiveness in stages 4 and 5 could be caused by the relatively large number of remaining CFUs from earlier stages.

Recently we reported on the ability of *S. aureus* to develop a resistance to an otherwise effective dose of blue light (464-nm).[15] We designed this current experiment to consider not only whether the light energy would effectively inhibit *C. albicans*, but also to see whether the organism would demonstrate signs (loss of kill rate effectiveness) of resistance formation across successive stages of the experiment. These data suggest *C. albicans* has this ability (see downward kill rate trend stages 4 and 5), but that the issue can be reversed by lowering the rate of energy delivery (stages 6 and 7). This lowered rate of delivery was not effective with the 9 J/cm^2 dose, but improved the outcome for 30 J/cm^2. It is our opinion that the improved effectiveness (compared to stages 4 and 5) of the treatment in later stages was facilitated by better absorption of the light energy. That is to say, when the energy is applied at a low rate there is a greater opportunity for the organism to absorb the entire dose. Had we employed the lower rate of delivery in the earlier stages of the experiment we could have seen even greater inhibition in those stages.

The outcomes from this experiment suggest a foundation for developing a clinical treatment protocol for wounds infected by *C. albicans*. Since patients would likely be treated over time with successive energy deliveries, we believe an approach that combines wavelengths and varies rate of energy delivery is supported. Based on this work, no more than three or four light deliveries should be done without a change in rate of delivery.

CONCLUSIONS

Based on the data collected in this experiment, we would draw the following conclusions.

1. Combining 624 and 850-nm wavelengths is a potentially effective approach to inhibiting *C. albicans* in vitro.
2. 30 J/cm^2 is a more effective dose for this organism.
3. Varying the rate of energy delivery may improve outcomes over successive applications of the light energy.

Additional research is needed to identify the best rate of energy delivery for the purposes of microbial inhibition. We have begun this work and are experimenting with rates from 5 mW/cm^2 to 125 mW/cm^2.

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